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Purification and Some Properties of a Soluble Benzene-Oxidizing System from a Strain of *Pseudomonas*

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1. A soluble enzyme system which oxidizes benzene to *cis*-1,2-dihydroxycyclohexa-3,5-diene (*cis*-benzene glycol) was obtained from a species of *Pseudomonas* grown on benzene as the major carbon source. 2. The system was shown to consist of three protein components. Two of these were non-haem-iron proteins of molecular weight approx. 21000 and approx. 186000 and the other was a flavoprotein of molecular weight approx. 60000. 3. Fe^{2+} and NADH were essential cofactors for benzene oxidation.

Gibson *et al.* (1968) demonstrated that a soluble system capable of benzene oxidation was present in cell-free extracts of a strain of *Pseudomonas putida* grown with toluene as the sole source of carbon and energy. These authors were able to resolve the system into two fractions by $(NH_4)_2SO_4$ precipitation, both of which were necessary for benzene oxidation. Högn & Jaenicke (1972) also obtained two separate fractions from extracts of a *Moraxella* species which, when combined, had the ability to oxidize benzene. Further attempts to purify these fractions were unsuccessful.

Samples of soil were screened in our laboratories to isolate organisms that utilize benzene for growth. Several species of *Arthrobacter* and *Pseudomonas* were obtained which grew on benzene added directly to the aqueous culture medium. Cell-free extracts of the *Arthrobacter* species had benzene dioxygenase systems that were extremely unstable. A *Pseudomonas* species identified as *Pseudomonas putida* was isolated which grew rapidly in good yield on benzene in a simple salts medium, and was found to have a soluble benzene dioxygenase system which was relatively stable for several weeks at 0–4°C (Axcell & Geary, 1973). Our studies have shown that this system may be separated into three protein fractions, each of which has been obtained in an essentially pure form, and all of which are required for benzene dioxygenase activity.

Materials and Methods

Methods

Growth and maintenance of the organism. Large-scale growth of biomass was performed by the method of Axcell & Geary (1973). Cells were cultured in Biotec 10-litre fermenters (Biotec AB, Bromma, Sweden) in a simple salts aqueous medium maintained at 30°C and pH 7.2, to which benzene was added at a controlled rate with the incoming air. Stock cultures were maintained on agar slopes prepared in screw-cap bottles. The organism was sub-cultured monthly and stored at 4°C.

Preparation of cell-free extracts. Washed cells (100g) were thawed and resuspended in 100ml of buffer 1 (25mM-potassium phosphate, pH 7.4). The suspension was cooled in ice and subjected to the maximum output from a Branson Soniprobe ultrasonic disruptor (Dawe Instruments Ltd., London W.3, U.K.). Batches (50ml) were treated for six periods of 30s each. The probe was cooled in ice between periods of use. Cell debris and unbroken cells were removed by centrifugation at $40000g_{av.}$ for 1h. The supernatant solution, containing 70–80mg of protein/ml, was decanted. These and all other stages in the purification were performed between 0° and 4°C. All centrifugations were done in an MSE 10 × 100ml rotor. Fractions obtained during the purification were stored at −22°C except where specified otherwise.

Standard assay. O_2 consumption in the presence of benzene was measured at 30°C with a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.). Reaction mixtures contained a suitable amount of protein, 0.5μmol of NADH, 0.3μmol of $FeSO_4$, 100μg of partially purified catechol 1,2-dioxygenase and 100μg of *cis*-1,2-dihydroxycyclohexa-3,5-diene (NAD^+) oxidoreductase (*cis*-benzene glycol dehydrogenase), in a total volume of 3ml of buffer 1. The endogenous rate of O_2 consumption was measured in the absence of substrate and the reaction was initiated by the addition of 20μl of a 10mM solution of benzene in water. The rate of oxygen consumption increased initially with time and the reaction rate was thus measured on the linear part of the curve at 50% air-saturation. If the enzyme was allowed to exhaust the substrate and the reaction mixture was re-aerated to

the original O_2 concentration, a further addition of substrate resulted in a faster rate of reaction. The maximum rate was achieved when this process was repeated twice. Catechol 1,2-dioxygenase and *cis*-benzene glycol dehydrogenase were added as a routine to these assays to increase reaction rates and make them comparable with those of crude extracts. O_2 concentrations in air-saturated solutions were calculated by the method of Glasstone (1946).

Spectrophotometry. All measurements were made in 10mm light-path cuvettes by using a Pye–Unicam SP.1800 recording spectrophotometer.

Ultracentrifuge measurements. A Beckman model E analytical ultracentrifuge (Beckman-RIIC Ltd., Glenrothes, Fife, U.K.) was used to obtain sedimentation-velocity and molecular-weight data. The sedimentation coefficient (s) was determined by using a double-sector cell. Molecular weights were determined, by using interference optics, by the meniscus-depletion method of Yphantis (1964). The partial specific volume ($\bar{v}$) was calculated from the amino acid composition of the protein by the method of Schachman (1957). All measurements of photographic plates were made with a Projectorscope travelling microscope (Precision Grinding Ltd., Mitcham, Surrey, U.K.).

Polyacrylamide-gel electrophoresis. Each purified component of the system was examined by polyacrylamide-disc-gel electrophoresis (Shandon Scientific Co. Ltd., London N.W.10, U.K.) by the method of Williams & Reisfeld (1964). Sample and large-pore gels were at pH 5.5 and the small-pore separating gel was at pH7.5. Tris (1g/l)–barbitone (5.52g/l) buffer, pH7.0, was used in both reservoirs. Electrophoresis was performed at room temperature with a constant current of 4mA/tube until the Bromophenol Blue marker was 5mm from the lower end of the gel. Protein was stained by immersing the gels in 1% (w/v) Amido Black (George T. Gurr Ltd., London N.W.9, U.K.) in 7% (v/v) acetic acid for 1h. Excess of dye was removed by immersion in 7% (v/v) acetic acid for 48h. Relative band densities were measured with a Joyce–Loebl u.v. scanner (Joyce–Loebl and Co., Gateshead, Co. Durham, U.K.).

Determination of iron and inorganic sulphur. The iron content of individual proteins was determined by the spectrophotometric method of Rajagopalan & Handler (1964) by using published values for the extinction coefficients at about 550, 450 and 330nm per g-atom of iron, and was confirmed by atomic absorption spectroscopy. Inorganic sulphur was determined by the method of Siegel (1965).

Amino acid analysis. Proteins for amino acid analysis were hydrolysed in 6M-HCl at 128°C under N_2 for 24h and their amino acid compositions were determined by using a Technicon NC-1 Amino Acid Analyzer (Technicon Instruments Co. Ltd., Hanworth Lane, Chertsey, Surrey, U.K.).

Electron-paramagnetic-resonance (e.p.r.) spectroscopy. E.p.r. spectroscopy was performed by using a Varian E-line e.p.r. spectrometer with a 22.9cm (9in) magnet (Varian AG, Steinhauserstrasse, 6300 Zug, Switzerland). The sample temperature was controlled with a liquid helium cryostat.

Purification of the benzene dioxygenase system. Soluble extracts of cells grown on benzene were treated with protamine sulphate and brought to 40% (w/v) saturation with $(NH_4)_2SO_4$ as described by Axcell & Geary (1973). The supernatant solution obtained after collection of the 0–40% (w/v) $(NH_4)_2SO_4$ precipitate was adjusted to 70% (w/v) saturation by the addition of a saturated solution of $(NH_4)_2SO_4$, pH7.4. The mixture was stirred for 15min and the precipitate was collected by centrifugation at 40000$g_{av.}$ for 30min. The 40–70% (w/v) $(NH_4)_2SO_4$ precipitate was dissolved in the minimum volume of buffer 1 and dialysed for 18h against 2 litres of the same buffer.

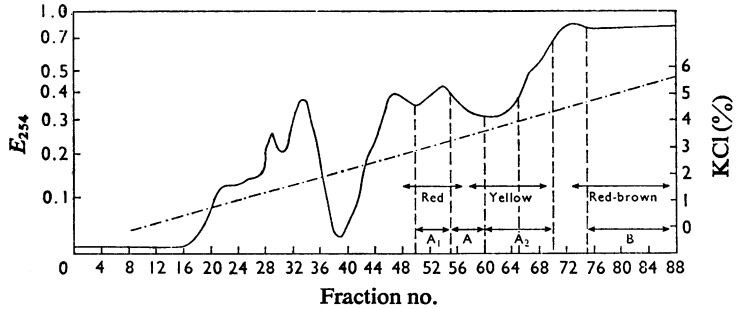


Fig. 1. *Elution of protein from DEAE-cellulose by using a linear 0–6% (w/v) KCl gradient in 1 litre of 25-mM-potassium phosphate buffer, pH7.4*

For details see the text. -·-·-, KCl gradient; ——, protein (E_{254}).

Table 1. *Assay of concentrated fractions from the DEAE-cellulose column*

The standard assay was used, samples (200 μl) from each fraction being added as shown. For details see the text.

Fractions added	Final rate (μmol of O_2/min)
B	0
A	0
B + A	0.171
B + A1	0.019
B + A2	0.023
B + A1 + A2	0.138

The dialysed 40–70% (w/v) $(NH_4)_2SO_4$ fraction was applied to the top of a column (1260 mm^2 × 420 mm) of DEAE-cellulose (Whatman DE-52; Whatman Biochemicals Ltd., Maidstone, Kent, U.K.) previously equilibrated with buffer 1. Proteins were eluted by means of a linear 0–6% (w/v) KCl gradient in 1 litre of the same buffer. Fractions (12 ml) were collected at 12 min intervals (Fig. 1). A direct assay of the individual fractions proved impossible owing to the high protein concentration required in the reaction mixture for benzene oxidation. The fractions were therefore concentrated in consecutive groups of five by ultrafiltration, by using an Amicon PM-10 membrane (Amicon, High Wycombe, Bucks., U.K.), to a final volume of 5–6 ml in each case. Combinations of two of these concentrates were tested for activity in the standard assay. Activity was found when samples (200 μl) from area B were assayed in the presence of a sample (200 μl) from area A (fractions 56–60). Area A was located between two strongly coloured protein bands, A1 (fractions 51–55), which was red, and A2 (fractions 61–70), which was yellow. Concentrated samples from these two areas had low activity when assayed singly in the presence of samples from area B, but when samples from areas A1, A2 and B were combined in the assay, full activity was again demonstrated (Table 1). Catechol 1,2-dioxygenase was located in area A2, but partially purified preparations of this enzyme from the 0–40% (w/v) $(NH_4)_2SO_4$ fraction would not substitute for the A2 sample in the benzene dioxygenase assay. Since the two active constituents in area A1–A–A2 were poorly resolved, fractions from this area were pooled and concentrated further by using an Amicon PM-10 membrane. Concentrates from area B which showed activity in the presence of those from area A were also pooled and reconcentrated to 5–6 ml.

Separation of proteins A1 and A2. The concentrated protein solution from area A1–A–A2 (8 ml) was applied to the top of a column (700 mm^2 × 600 mm) of Bio-Gel A 0.5 m (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) equilibrated with buffer 1, and proteins were eluted with the same buffer. Fractions (9 ml) were collected at 20 min intervals (Fig. 2). Well-de-

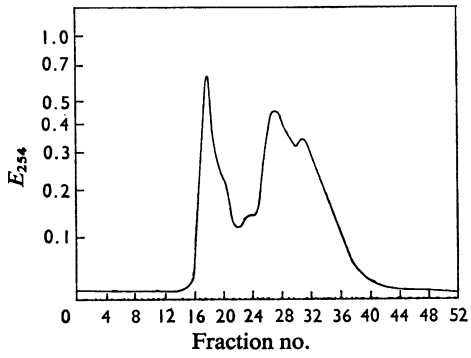


Fig. 2. *Separation of proteins A1 and A2 by chromatography on Bio-Gel A 0.5 m*

Protein A1 was obtained by concentrating fractions 26–29. Protein A2 was obtained from fractions 30–40. For details see the text.

fined red and yellow protein bands were eluted (proteins A1 and A2 respectively). These were shown to correspond to the components of the benzene dioxygenase complex by concentrating fractions from each band in groups of three, to 5 ml (approx.) and assaying a sample (200 μl) of each concentrate in the presence of a concentrated sample (200 μl) from the centre of the other band and a concentrated preparation containing protein B (200 μl), by using the standard assay system. Fractions showing activity were pooled and reconcentrated, yielding separate preparations of proteins A1 and A2.

Purification of protein A1. The concentrated preparation of protein A1 (8–9 ml) from the previous stage was centrifuged for 5 h at 4°C in an MSE 10 × 10 ml rotor at 150000$g_{av.}$. At the end of this time protein A1 was concentrated as a red pellet at the bottom of the centrifuge tube. The straw-coloured supernatant, which showed no benzene dioxygenase activity, was carefully removed and buffer 1 was added to dilute the red pellet to the original volume. The procedure was repeated and protein A1 was finally diluted to approx. 7 ml with buffer 1, yielding a preparation which appeared to be almost homogeneous in the analytical ultracentrifuge.

Purification of protein A2. Catechol 1,2-dioxygenase was removed from the concentrated preparation of protein A2 by passage through a column (380 mm^2 × 60 mm) of Bio-Gel HTP hydroxyapatite (Bio-Rad Laboratories) equilibrated in 10 mM-potassium phosphate buffer, pH 7.4. Proteins were eluted with a linear gradient of potassium phosphate (300 ml) from 10 to 400 mM, pH 7.4. Fractions (5 ml) were collected at 20 min intervals (Fig. 3). Protein A2 was located by the standard assay after concentration of the fractions. Catechol 1,2-dioxygenase was found

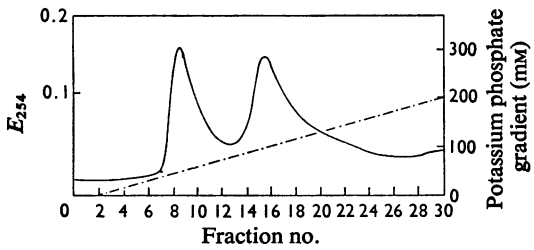


Fig. 3. *Elution of protein A2 from Bio-Gel HTP by using a 300 ml linear gradient of potassium phosphate* (10–400 mM, *pH* 7.4)

For details see the text. -·-·-, Potassium phosphate gradient; ——, protein (E_{254}).

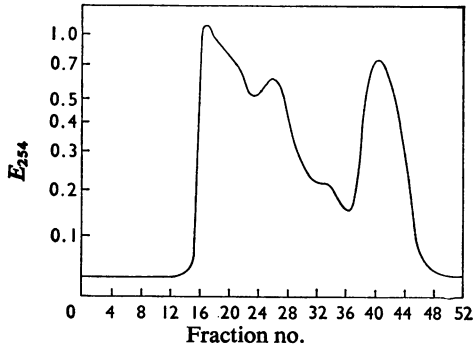


Fig. 4. *Elution of protein B from Bio-Gel A* 0.5 *m*

Protein B was obtained by concentrating fractions 33–37. For details see the text.

to be in the initial peak of protein. Fractions having activity in the benzene dioxygenase assay were pooled and reconcentrated to 5 ml (approx.) (13–19).

Purification of protein B. The concentrated preparation of protein B (8 ml) from the DEAE-cellulose column was applied to the top of the Bio-Gel A 0.5 m column described above. Proteins were eluted in the same manner (Fig. 4). Protein B was located by concentrating and assaying groups of fractions and the combined active fractions were further concentrated to 2–3 ml and applied to the top of a column (500 mm^2 × 450 mm) of Sephadex G-75 (Pharmacia Fine Chemicals AB, Uppsala, Sweden) equilibrated in buffer 1. Proteins were eluted with the same buffer, fractions (5 ml) being collected at 17 min intervals (Fig. 5). Protein B was again located and active fractions were pooled and reconcentrated to 5 ml (approx.).

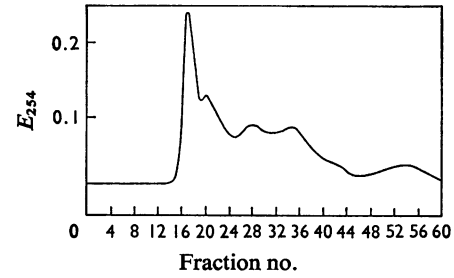


Fig. 5. *Elution of protein B from Sephadex G-75*

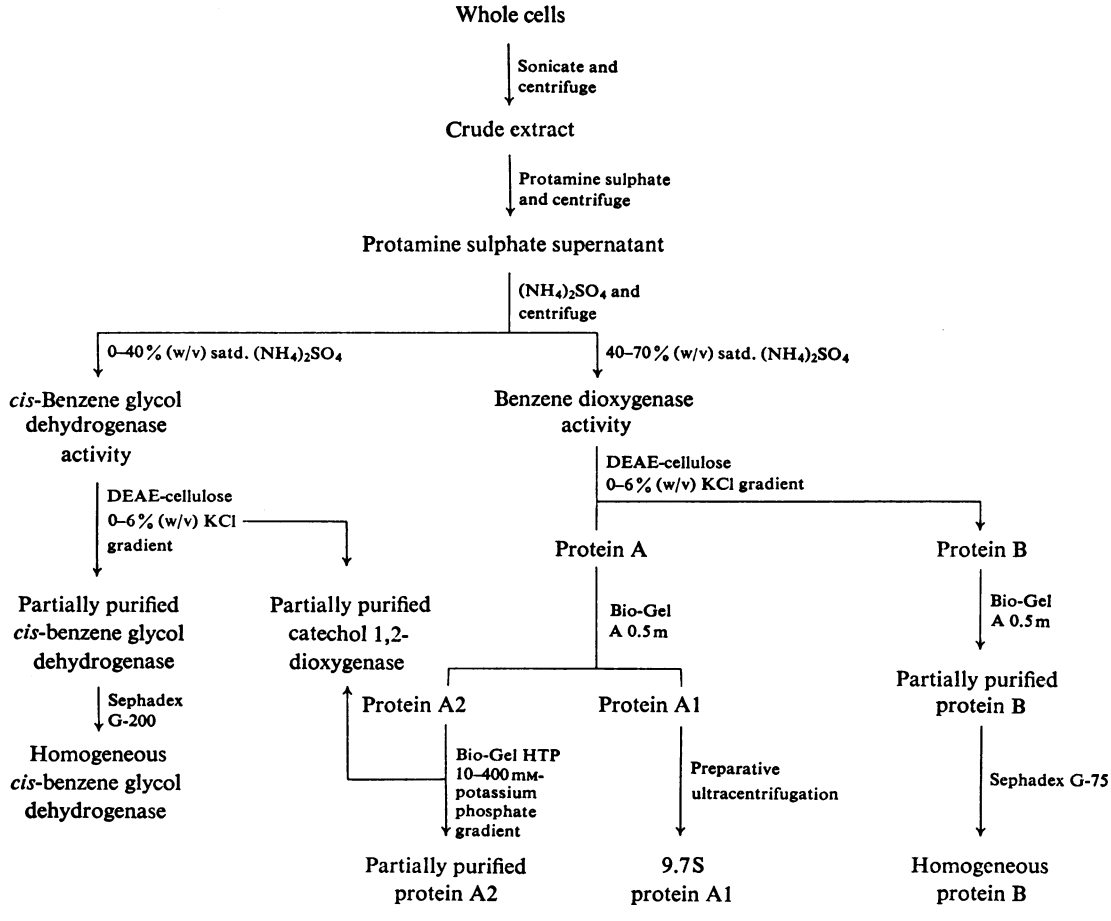
Protein B was obtained by concentrating fractions 33–39. For details see the text.

Table 2. *Summary of the purification of components of the benzene dioxygenase system*

One unit is defined as the amount of protein which catalyses the consumption of 1 nmol of O_2/min in the standard assay system. Note that values quoted for protein B represent a different purification run to those recorded for proteins A1 and A2.

Protein	Stage	Volume (ml)	Activity (units)	Protein (mg)	Specific activity (units/mg of protein)	Recovery (%)
A	Crude cell-free extract	65	70500	5720	12.3	100
	Protamine sulphate supernatant	70	73000	5610	13.0	100
	40–70% (w/v) satd. $(NH_4)_2SO_4$ fraction (dialysed)	52	41000	1708	24.0	58
A1	DEAE-cellulose eluate	10*	35500	440	81.0	50
	Bio-Gel A 0.5 m eluate	9	14000	117	120.0	19
	Ultracentrifuge 9.7S fraction	7	8250	58	142.0	12
A2	DEAE-cellulose eluate	14*		228	84.0	27
	Bio-Gel A 0.5 m eluate	11		20	440.0	12
	Bio-Gel HTP eluate	5		15	320.0	7
B	Crude cell-free extract	140		6720	17.0	100
	Protamine sulphate supernatant	145		6480	17.5	100
	40–70% (w/v) satd. $(NH_4)_2SO_4$ fraction (dialysed)	70		2980	33.2	87
	DEAE-cellulose eluate	18		545	69.0	33
	Bio-Gel A 0.5 m eluate	9		31	525.0	14
	Sephadex G-75 eluate	5		9	780.0	6

* These fractions were recombined and concentrated before chromatography on Bio-Gel A 0.5 m.



Scheme 1. *Purification of the enzymes responsible for the initial metabolism of benzene*

The purification of the benzene dioxygenase system is summarized in Table 2 and Scheme 1. For the purposes of consistency each component was assayed with a constant amount (200μl of concentrated solution) of each of the other two components at the Bio-Gel A 0.5m stage of purification. At this stage in the purification all three components were required for activity in the benzene dioxygenase assay and the activity was zero if any one of the components was omitted. Protein was measured by the method of Lowry *et al.* (1951). A standard curve was prepared by using a dry sample of bovine serum albumin.

Materials

cis-Benzene glycol dehydrogenase and catechol 1,2-dioxygenase were prepared as described by Axcell & Geary (1973). *cis*-Benzene glycol was prepared by the method of Nakajima *et al.* (1956). Materials used for disc electrophoresis were supplied by Kodak Ltd., Kirkby, Liverpool, U.K. NAD^+, NADH, NADPH, protamine sulphate and bovine serum albumin were from Sigma (London) Ltd., Kingston-upon-Thames, Surrey, U.K. Catechol, FMN and FAD were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. All other reagents were supplied by BDH Chemicals Ltd., Poole, Dorset, U.K., except where stated in the text.

Results

Substrate specificity

The 40–70% (w/v) $(NH_4)_2SO_4$ fraction, after dialysis, was shown to catalyse the consumption of O_2 in the standard assay system in the presence of a range of substrates including toluene, fluorobenzene,

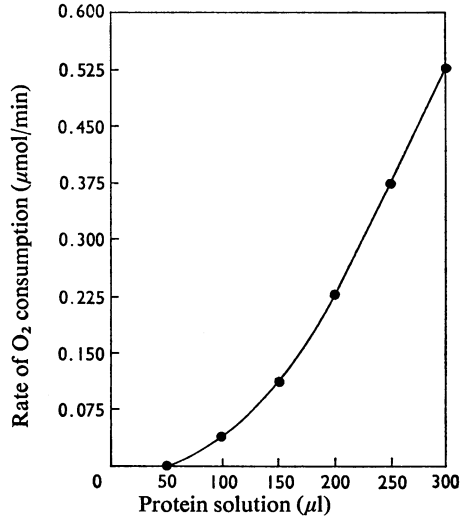


Fig. 6. *Dependence of reaction rate on protein concentration*

The standard assay was used. Dialysed 40–70% (w/v) satd. $(NH_4)_2SO_4$ fraction containing 33mg of protein/ml was added as indicated.

chlorobenzene, bromobenzene and iodobenzene. Substrates were supplied as saturated solutions in distilled water, 20μl of each solution being supplied to the assay, which contained 7mg of protein. Benzoic acid, benzaldehyde, acetophenone, phenol, *p*-xylene and naphthalene were not metabolized under these conditions.

Effect on the reaction rate of increasing protein concentration

At relatively low protein concentrations the enzyme system showed a non-linear dependence of reaction rate on protein concentration (Fig. 6). The amounts of protein used in assays were therefore selected to ensure as far as possible that the rate obtained was on the linear portion of the curve of reaction rate against protein concentration at each stage in the purification.

Formation of product

Purified preparations of the benzene dioxygenase system which were not contaminated with *cis*-benzene glycol dehydrogenase were allowed to react with benzene in the standard assay mixture. *cis*-Benzene glycol dehydrogenase was omitted from these reaction mixtures until the benzene was exhausted and the reaction mixture had been re-aerated. On addition of pure *cis*-benzene glycol dehydrogenase a further uptake of O_2 was observed due to the conversion of *cis*-benzene glycol into *cis,cis*-muconic acid via catechol (Axcell & Geary, 1973).

Cofactor requirements

Benzene dioxygenase activity could not be measured in the absence of Fe^{2+}. $FeSO_4,7H_2O$ was therefore added to all reaction mixtures at a final concentration of 0.1mM. NADH was required as the electron donor for the system. Benzene dioxygenase activity could not be shown when NADPH, at the same concentration, was substituted for NADH.

Ultracentrifuge measurements and polyacrylamide-gel electrophoresis

Protein A1. Sedimentation-velocity analysis of protein A1 from the Bio-Gel A 0.5m column stage showed two components of $s_{20,w}$ $5.8 \times 10^{-13}s^{-1}$ and $s_{20,w}$ $9.7 \times 10^{-13}s^{-1}$ (Fig. 7*a*). This protein appeared almost homogeneous by polyacrylamide-gel electrophoresis (Fig. 8), and further chromatography on ion-exchange cellulose and gel-filtration columns failed to resolve the two components visible in the analytical ultracentrifuge. The components were separated by preparative ultracentrifugation, as described under 'Methods', yielding a preparation containing mainly the 9.7S species which was active when tested in the benzene dioxygenase assay (Fig. 7*b*). When this solution was frozen and thawed once the presence of a higher-molecular-weight species was demonstrated and there was a decrease in the residue of 5.8S material (Fig. 7*c*). A molecular weight of approx. 186000 was obtained for a freshly prepared sample of the 9.7S material by the meniscus-depletion method of Yphantis (1964). The rotor was operated at 12000rev./min at 16°C. The protein concentration was 1mg/ml in buffer 1 and this buffer was used in the reference side of the double-sector centrepiece. The partial specific volume ($\bar{v}$) was calculated as 0.732 ml/g from the amino acid composition of the protein.

Protein A2. A preparation of protein A2, obtained by concentrating fractions from the Bio-Gel column, yielded four main bands on polyacrylamide-gel electrophoresis, none of which corresponded to those obtained with protein A1 or B, and was observed to sediment as a broad single peak when subjected to sedimentation-velocity analysis. The average *s* value was calculated as $4.2 \times 10^{-13}s^{-1}$, corresponding to a molecular weight of approx. 60000 (Chervenka, 1969). Further attempts at purification caused a partial loss of colour and an increase in the number of bands obtained on electrophoresis gels, indicating that this protein is unstable in its purified form.

Protein B. A preparation of protein B which appeared to be almost homogeneous by polyacrylamide-gel electrophoresis (Fig. 9) was subjected to sedimentation-velocity analysis at 60000rev./min at 20°C

yielding an $s_{20,w}$ value of $2.3 \times 10^{-13} s^{-1}$. A molecular weight of approx. 21000 was obtained by the method of Yphantis (1964). The rotor was operated at 40000 rev./min at 23.6°C. The protein concentration was 1 mg/ml in buffer 1 and this buffer was also used in the reference side of the double-sector centrepiece. A value for $\bar{v}$ of 0.73 ml/g was obtained from the amino acid composition of the protein.

Amino acid analysis

The amino acid compositions of proteins A1 and B are given in Table 3.

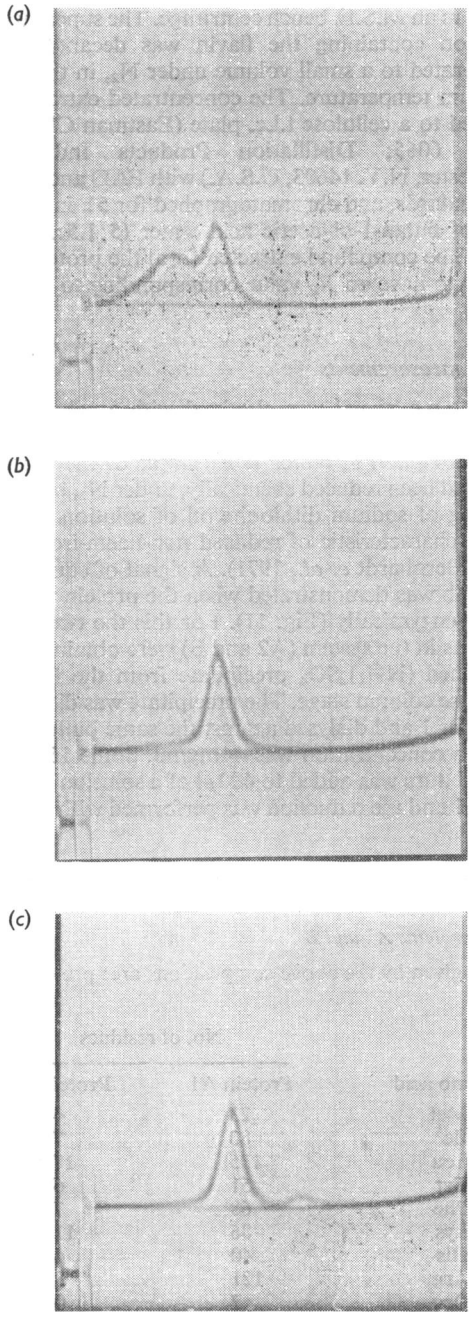


Fig. 7. *Ultracentrifuge patterns obtained with protein A1*

(*a*) Protein A1 after elution from Bio-Gel A 0.5m. The protein concentration was 9mg/ml. (*b*) Protein A1 after centrifuging to remove the light component. The protein concentration was 8mg/ml. (*c*) Protein A1 after removal of the light component and freezing and thawing once. The protein concentration was 8mg/ml. The photographs were taken 32min after the rotor had reached two-thirds of operating speed (60000rev./min). The temperature was 20°C and the phase-plate angle was 65°. Sedimentation was from left to right in 25mM-potassium phosphate buffer, pH7.4.

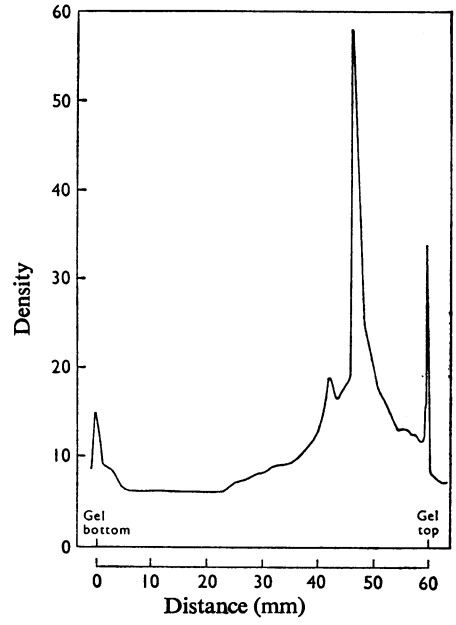


Fig. 8. *Polyacrylamide-gel electrophoresis of protein A1*

Densitometer trace obtained from a stained gel after electrophoresis of 100μg of protein. Electrophoresis was performed at room temperature with a constant current of 4mA/tube until the Bromophenol Blue marker was 5mm from the lower end of the gel. Protein was stained by immersing the gels in 1% (w/v) Amido Black in 7% (v/v) acetic acid for 1h and excess of dye was removed by immersion in 7% (v/v) acetic acid for 48h. Relative band densities were measured with a Joyce–Loebl u.v. scanner.

Determination of iron and inorganic sulphur

The absorption spectra of proteins A1 (Fig. 10*b*) and B (Fig. 10*c*) suggested that both contained non-haem iron. Protein A1 was found to contain 2 atoms of iron and 3 atoms of inorganic sulphur/mol of mol. wt. 186000, whereas protein B contained 1 atom of iron and between 1 and 2 atoms of inorganic sulphur/mol of mol. wt. 21000.

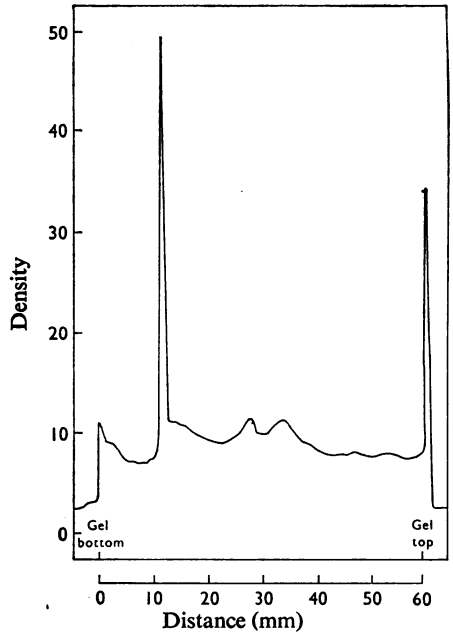


Fig. 9. *Polyacrylamide-gel electrophoresis of protein B*

For details see Fig. 8.

Identification of the flavin component of protein A2

Protein A2 was yellow in colour and its spectrum was characteristic of that of a flavoprotein (Mahler & Cordes, 1966) (Fig. 10*a*). The flavin component was extracted by mixing 1ml of a solution (10mg/ml) of the protein with 4ml of an ice-cold 50:50 (v/v) ethanol–methanol mixture. The precipitated protein was removed by centrifuging for 10min at maximum speed in an M.S.E. bench centrifuge. The supernatant solution containing the flavin was decanted and evaporated to a small volume under N_2, in the dark at room temperature. The concentrated extract was applied to a cellulose t.l.c. plate (Eastman Chromagram 6065; Distillation Products Industries, Rochester, N.Y. 14603, U.S.A.) with FAD and FMN as standards, and chromatographed for 5h in a mixture of butan-1-ol–acetic acid–water (5:1.5:3.2, by vol.). The compound extracted from the protein was found to have an R_F value corresponding to that of FAD.

E.p.r. measurements

No e.p.r. signal was obtained with a solution of protein A1 (47mg/ml of concentrated Bio-Gel column eluate) in buffer 1. A sample of the protein that had been reduced chemically, under N_2, by using 16.5mg of sodium dithionite/ml of solution gave a signal characteristic of reduced non-haem-iron proteins (Bernhardt *et al.*, 1971). A signal of equivalent strength was demonstrated when the protein was reduced enzymically (Fig. 11). For this the two other proteins in the system (A2 and B) were obtained as a combined $(NH_4)_2SO_4$ precipitate from the DEAE-cellulose column stage. The precipitate was dissolved in buffer 1 and dialysed against the same buffer. The protein concentration was 44mg/ml. Some 150μl of this mixture was added to 400μl of a solution of protein A1 and the reduction was performed with 1μmol

Table 3. *Amino acid compositions of proteins A1 and B*

Tryptophan was not determined. The approximate molecular weights given by the above compositions are: protein A1, 181 000; protein B, 21 500.

Amino acid	No. of residues		Amino acid	No. of residues	
	Protein A1	Protein B		Protein A1	Protein B
Asx	170	22	Met	23	4
Thr	89	12	Ile	83	7
Ser	77	10	Leu	135	17
Glx	185	27	Tyr	51	6
Pro	70	10	Phe	68	7
Gly	115	20	Lys	86	11
Ala	166	18	His	40	4
Val	103	15	Arg	121	8
Cys	13	2	Orn	7	0

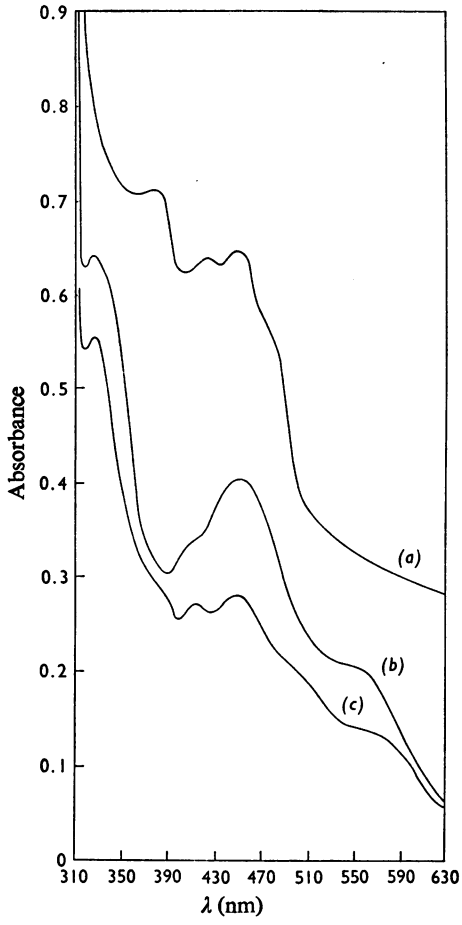


Fig. 10. *Spectra of components of the benzene dioxygenase system in 25mM-potassium phosphate buffer, pH7.4*

(*a*) Protein A2; the protein concentration was approx. 2mg/ml. (*b*) Protein A1; the protein concentration was 9.2mg/ml. (*c*) Protein B; the protein concentration was 1.2mg/ml.

of NADH at 30°C for 5min. Under the same conditions and at equivalent protein concentrations no reduction of protein A1 was observed with NADH alone. A small signal (12% of that obtained with the complete system) was obtained with the mixture of proteins A2 and B and NADH, which may be attributed to the presence of a small amount of protein A1 in this preparation.

In the presence of Fe^{2+} (1mM), which is normally required for the conversion of benzene into *cis*-benzene glycol by the enzyme system, no e.p.r. signal was detected from protein that had been reduced either enzymically or chemically. However, the optical absorption spectra of these preparations indicated that reduction of the protein had occurred (Fig. 12).

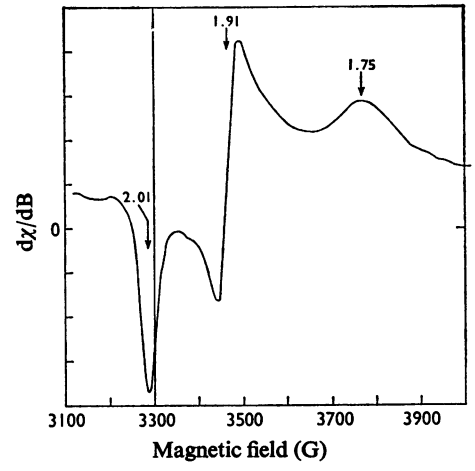


Fig. 11. *E.p.r. spectrum of enzymically reduced protein A1*

The concentration of protein A1 was 34mg/ml. Modulation amplitude, 0.63×10^1G; receiver gain, 3.2×10^3; microwave power, 56mW; modulation frequency, 100kHz; microwave frequency, 9.25593GHz; temperature, 70°K.

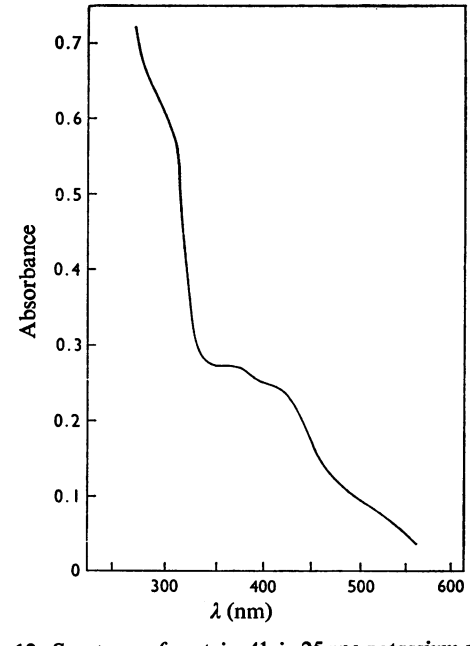
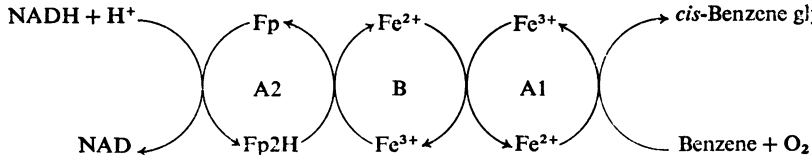


Fig. 12. *Spectrum of protein A1 in 25mM-potassium phosphate buffer, pH7.4, after reduction with dithionite*

The protein concentration was 8.6mg/ml. A similar spectrum was obtained from protein A1 after reduction by NADH with proteins A2 and B.



Scheme 2. *Suggested functions of components of the benzene dioxygenase system*

Fp, Flavoprotein.

Function of individual proteins in the system

In the presence of protein A2 alone, the oxidation of NADH by 2,6-dichlorophenol-indophenol can be demonstrated by measuring the decrease in E_{600}. The K_m value for this protein under these conditions, calculated from a double-reciprocal plot (Lineweaver & Burk, 1934) was 11.2 μM-NADH. Reaction mixtures contained 50μl of 200μM-dichlorophenol-indophenol, 30μg of protein and buffer 1 to a final volume of 1 ml. The reaction was initiated by addition of a suitable amount of NADH in each case. The oxidation of NADPH under the same conditions was less than 10% that of NADH. Oxidation of NADH by cytochrome *c* in the presence of protein A2 was negligible unless protein B was also present, when a decrease in the absorbance of the cytochrome at 550nm could be measured. Reaction mixtures contained 100μl of 1mM-cytochrome *c*, 30μg of protein A2, 100μg of protein B, 1μmol of NADH and buffer 1 to a final volume of 1 ml.

Discussion

Resolution of the three protein components of the benzene dioxygenase system has revealed similarities to mono-oxygenase systems already described (Bernhardt & Staudinger, 1973; George & Griffith, 1959). It has, however, been demonstrated that the benzene-oxidizing system is a true dioxygenase and that both atoms of O_2 incorporated into *cis*-benzene glycol derive from a single O_2 molecule (Gibson *et al.*, 1970).

Our results indicate that the red protein, A1, of mol.wt. 186000 is the terminal dioxygenase. The appearance of an e.p.r. signal at $g = 2$ (approx.) on reduction of the protein enzymically or chemically is thought to be due to the reduction of one of a pair of anti-ferromagnetically coupled Fe^{III} atoms. Initially no e.p.r. signal due to Fe^{III} would be seen from this couple, but on reduction of one Fe^{III} to Fe^{II} the typical signal would be observed (Aasa, 1970). The effect of added Fe^{2+} on this system, causing the loss of the e.p.r. signal from the reduced protein, but not apparently affecting the optical spectrum, has not yet been explained.

The instability of flavoprotein A2 in its purified form has so far made accurate measurements difficult with this component. The small non-haem-iron protein B was obtained in a homogeneous state and is relatively stable. By analogy with mono-oxygenase systems we suggest that protein A2 functions as the NADH–protein B oxidoreductase (Scheme 2).

A marked concentration-dependence was shown by the system during assays of crude and purified preparations. It was also found that the amount of protein required for activity was relatively high. Enzyme activity increased when the same assay mixture was incubated with successive portions of substrate. This effect became more noticeable as the components of the system were purified, and it is suggested that some organization of the components and cofactors is necessary for activity towards benzene.

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